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(54) **Method of producing L-glutamic acid by fermentation.**

(57) The present invention provides a low-cost and efficient production method of L-glutamic acid, by improving the productivity of L-glutamic acid in a microorganism of the genus Escherichia.  
[Constitution]

A method of producing L-glutamic acid by fermentation comprising culturing in a liquid culture medium a mutant of the genus Escherichia having L-glutamic acid-producing ability whose  $\alpha$ -ketoglutarate dehydrogenase activity is deficient or reduced and phosphoenolpyruvate carboxylase and glutamate dehydrogenase activities are amplified, accumulating L-glutamic acid in the culture, and recovering L-glutamic acid therefrom.

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## Field of the Invention

The present invention relates to a mutant useful for producing L-glutamic acid by fermentation as well as a method of producing L-glutamic acid by fermentation using such a mutant. L-glutamic acid is an amino acid widely used as an additive for foods and in medicaments.

## Prior Art

L-glutamic acid has conventionally been produced by fermentation using glutamic acid-producing bacteria and mutants thereof such as those of the genus Brevibacterium, Corynebacterium or Microbacterium (Amino acid fermentation, Gakkai Shuppan Center, pp.195 to 215 (1986)). Other known methods of producing L-glutamic acid by fermentation include a method employing microorganisms of the genus Bacillus, Streptomyces or Penicillium (US Patent No. 3,220,929) and a method employing microorganisms of the genus Pseudomonas, Arthrobacter, Serratia or Candida (US Patent No. 3,563,857). Even though such conventional methods produce significantly large amounts of L-glutamic acid, an even more efficient and less expensive method of producing L-glutamic acid is desired in order to meet the ever-increasing demand.

Escherichia coli is a potentially excellent L-glutamic acid-producing bacterium in view of its high growth rate and the availability of sufficient gene information, while the reported amount of L-glutamic acid production by Escherichia coli is as low as 2.3 g/l (J. Biochem., Vol. 50, pp.164 to 165 (1961)). Recently, a mutant exhibiting a deficient or reduced  $\alpha$ -ketoglutarate dehydrogenase (hereinafter referred to as  $\alpha$ -KGDH) was reported to have the ability to produce large amounts of L-glutamic acid (French Patent Application Laid-Open No. 2680178).

## Problems to be Solved by the Invention

An objective of the present invention is to enhance the L-glutamic acid-producing ability of strains belonging to the genus Escherichia and to provide a method of producing L-glutamic acid more efficiently and at a lower cost.

## Means to Solve the Problems

Now it has been found surprisingly in our study on the production of L-glutamic acid by mutants of Escherichia coli that a mutant whose  $\alpha$ -KGDH activity is deficient or reduced, and whose phosphoenolpyruvate carboxylase (hereinafter referred to as PPC) and glutamate dehydrogenase (hereinafter referred to as GDH) activities are enhanced, has a high L-glutamic acid-producing ability, and thus the present invention has been accomplished.

Accordingly, the present invention relates to :

A mutant of the genus Escherichia having L-glutamic acid-producing ability whose  $\alpha$ -KGDH activity is deficient or reduced, and PPC and GDH activities are enhanced; and,

A method of producing L-glutamic acid by fermentation comprising culturing in a liquid culture medium a mutant of the genus Escherichia having L-glutamic acid-producing ability whose  $\alpha$ -KGDH activity is deficient or reduced and PPC and GDH activities are enhanced, accumulating L-glutamic acid in the culture, and recovering L-glutamic acid therefrom.

The present invention is described in more detail below.

(1) Derivation of a mutant of the genus Escherichia exhibiting deficient or reduced  $\alpha$ -KGDH activity

As a starting parent strain to be used for preparing the present mutant, any non-pathogenic strain of the genus Escherichia may be employed. Examples of such strains are listed below.

Escherichia coli K-12 (ATCC 10798)

Escherichia coli W3110 (ATCC 27325)

Escherichia coli B (ATCC 11303)

Escherichia coli W (ATCC 9637)

A mutant of the genus Escherichia which has L-glutamic acid-producing ability and having deficient or reduced  $\alpha$ -KGDH activity may be prepared as follows.

The starting parent strain mentioned above is first exposed to X-radiation or ultraviolet light or mutagenic agents such as N-methyl-N'-nitro-N-nitrosoguanidine (hereinafter referred to as NG) to introduce the mutation.

Alternatively, gene engineering technology, for example, gene recombination, gene transformation or cell

fusion, may be used to efficiently introduce the intended mutation.

A method of obtaining an  $\alpha$ -KGDH-deficient mutant by means of gene recombination is conducted as follows. Based on the known nucleotide sequence (Euro. J. Biochem. Vol. 141, pp. 351 to 359 (1984)) of  $\alpha$ -ketoglutarate dehydrogenase gene (hereinafter referred to as sucA gene), primers are synthesized and then the sucA gene is amplified by the PCR method using the chromosomal DNA as a template. Into the amplified the sucA gene, a drug-resistant gene is inserted to obtain a sucA gene whose function is lost. Subsequently, using homologous recombination, the sucA gene on the chromosome is replaced by a sucA gene whose function is lost by means of the insertion of the drug-resistant gene.

After subjecting the parent strain to mutagenic treatment, the intended mutants may be screened by procedures as illustrated below.

A mutant exhibiting a deficient or reduced  $\alpha$ -KGDH activity is either not able to grow or is able to grow only at a significantly reduced growth rate in a minimum culture medium containing glucose as the carbon source under aerobic condition. However, even under such condition, normal growth is possible by adding succinic acid or lysine plus methionine to the minimum culture medium containing glucose. On the other hand, anaerobic condition allows the mutant to grow even in the minimum culture medium containing glucose (Molec. Gen. Genetics, Vol. 105, pp. 182 to 190 (1969)). Based on these findings, the desired mutants can be screened.

The following strain is an example of the mutants thus obtained whose  $\alpha$ -KGDH activity is deficient or reduced and which are listed below.

Escherichia coli W3110 sucA::Km<sup>r</sup>

A mutant whose  $\alpha$ -KGDH activity is deficient or reduced is more useful in view of its enhanced ability to produce L-glutamic acid when it further has the properties that L-glutamic acid-degrading activity is reduced or the expression of ace operon, that is, malate synthase (aceB) - isocitrate lyase (aceA) - isocitrate dehydrogenase kinase/phosphatase (aceK) operon becomes constitutive. These properties are discussed in French Patent Application Laid-open No. 2680178. As a matter of course, properties already known to be effective for improving L-glutamic acid-productivity, such as various types of auxotrophy, antimetabolite resistance and antimetabolite sensitivity, are also desirable for enhancing L-glutamic acid production ability.

A mutant having reduced ability to degrade L-glutamic acid may be isolated as a mutant which either cannot grow or can grow only slightly in a minimum culture medium containing L-glutamic acid as the sole carbon source instead of glucose or containing L-glutamic acid as a sole nitrogen source instead of ammonium sulfate. However, as a matter of course, when an auxotroph is employed for the derivation, the minimum essential amount of the nutrient required for the growth may be added to the culture medium.

A mutant in which the expression of the ace operon is constitutive may be obtained as a strain whose parent strain is a phosphoenolpyruvate synthase-deficient strain and which can grow in a minimum culture medium containing lactic acid as the carbon source but cannot grow in a minimum culture medium containing pyruvic acid or acetic/pyruvic acid as the carbon source, or as a strain which shows a higher growth rate than that of its parent strain whose  $\alpha$ -KGDH is deficient or reduced under aerobic condition (J. Bacteriol., Vol. 96, pp. 2185 to 2186 (1968)).

Examples of the mutants described above are as follows.

Escherichia coli AJ 12628 (FERM BP-3854)

Escherichia coli AJ 12624 (FERM BP-3853)

Escherichia coli AJ 12628 is a mutant having a reduced  $\alpha$ -KGDH activity and a reduced ability to degrade L-glutamic acid in combination with constitutive expression of ace operon. Escherichia coli AJ 12624 is a mutant having reduced  $\alpha$ -KGDH activity and a reduced ability to degrade L-glutamic acid (French Patent Application Laid-open No. 2680178).

In the mutant thus obtained which exhibits deficient or reduced  $\alpha$ -KGDH activity, the flow of biosynthesis of L-glutamic acid via  $\alpha$ -ketoglutaric acid in the TCA cycle is improved, resulting in an enhanced ability of producing L-glutamic acid. Also the productivity of L-glutamic acid is increased in the mutant exhibiting deficient or reduced  $\alpha$ -KGDH activity and significantly low ability to degrade the produced L-glutamic acid or in the mutant further having a constitutive expression of the ace operon whereby the growth is improved.

## (2) Derivation of a mutant of the genus Escherichia having amplified PPC activity and GDH activity

In the examples described below, a mutant of the genus Escherichia having amplified PPC and GDH activities was obtained from a starting parent strain exhibiting deficient or reduced  $\alpha$ -KGDH activity and having the ability to produce L-glutamic acid. It is also possible to use a wild strain of the genus Escherichia as the parent strain to obtain a mutant having amplified PPC and GDH activities whereafter a mutant is bred which exhibits deficient or reduced  $\alpha$ -KGDH activity.

Accordingly, the starting parent strain used to prepare a mutant having amplified PPC and GDH activities

is preferably a mutant of the genus Escherichia whose  $\alpha$ -KGDH activity is deficient or reduced and which has the ability to produce L-glutamic acid or a non-pathogenic wild type strain of the genus Escherichia. Examples of such strains are listed below.

- Escherichia coli W3100 sucA::Km<sup>r</sup>
- 5 Escherichia coli AJ 12628 (FERM BP-3854)
- Escherichia coli AJ 12624 (FERM BP-3853)

(Those listed above are the mutants of the genus Escherichia whose  $\alpha$ -KGDH activity is deficient or reduced and which have the ability to produce L-glutamic acid.)

- Escherichia coli K-12 (ATCC 10798)
- 10 Escherichia coli W3110 (ATCC 27325)
- Escherichia coli B (ATCC 11303)
- Escherichia coli W (ATCC 9637)

(Those listed above are the non-pathogenic wild strains of the genus Escherichia.)

- In order to amplify PPC and GDH activities, the genes coding for PPC and GDH are cloned in an appropriate plasmid, which is then used to transform the starting parent strain employed as a host. The copies of the genes coding for PPC and GDH (hereinafter referred to as ppc gene and gdhA gene, respectively) in the transformed cells are increased, resulting in amplified PPC and GDH activities.

- The ppc gene and gdhA gene to be cloned may be cloned into a single plasmid to be introduced into the starting parent strain employed as the host, or may be cloned separately into two types of plasmid which are compatible in the starting parent strain.

- Alternatively, the amplification of PPC and GDH activities may be conducted by allowing the ppc and gdhA genes to be present as multicopies on the chromosomal DNA of the starting parent strain employed as the host. In order to introduce the ppc and gdhA genes as multicopies into the chromosomal DNA of the genus Escherichia, homologous recombination is applied utilizing a target sequence present as a multicopy on the chromosomal DNA. The sequence present as the multicopy may be a repetitive DNA and an inverted repeat present at the terminal of insertion sequence. Alternatively, as described in Japanese Patent Application Laid-open No. 2-109985, the ppc and gdhA genes are cloned on a transposon, which is then transposed, thereby introducing the multicopy into the chromosomal DNA. The copies of the ppc and gdhA genes in the transformed cells are increased, resulting in the amplification of PPC and GDH activities.

- In addition to the gene amplification described above, the amplification of PPC and GDH activities may also be conducted by replacing the promoters of the ppc and gdhA genes with those having higher potencies. For example, lac promoter, trp promoter, trc promoter, tac promoter, P<sub>R</sub> promoter and P<sub>L</sub> promoter of a lambda phage are known to be strong promoters. By enhancing the expression of the ppc gene and of the gdhA gene, the PPC and GDH activities are amplified.

- The ppc and gdhA genes can be obtained by isolating the genes which are complementary with regard to auxotrophy of the mutants which are either PPC or GDH deficient. Alternatively, since the nucleotide sequences of these genes of Escherichia coli are known (J. Biochem., Vol. 95, pp. 909 to 916 (1984); Gene, Vol. 27, pp. 193 to 199 (1984)), the primers are synthesized based on the nucleotide sequences and then the genes are obtained by the PCR method using the chromosomal DNA as the template.

- (3) Production of L-glutamic acid by fermentation using a mutant of the genus Escherichia capable of producing L-glutamic acid which exhibits deficient or reduced  $\alpha$ -KGDH activity and has amplified PPC and GDH activities

- For the purpose of producing L-glutamic acid by fermentation using a mutant of the genus Escherichia capable of producing L-glutamic acid which exhibits deficient or reduced  $\alpha$ -KGDH activity and has amplified PPC and GDH activities, a standard culture medium containing carbon sources, nitrogen sources, inorganic salts and, if necessary, organic trace nutrients such as amino acids and vitamins and a standard culture method may be employed. The carbon sources and the nitrogen sources employed in the culture medium may be any of those catabolized by the mutant employed.

- The carbon sources may be saccharides such as glucose, glycerol, fructose, sucrose, maltose, mannose, galactose, starch hydrolysate and molasses, and organic acids such as acetic acid and citric acid may also be employed independently or in combination with other carbon sources.

- The nitrogen sources may be ammonia and ammonium salts such as ammonium sulfate, ammonium carbonate, ammonium chloride, ammonium phosphate, ammonium acetate as well as nitrates.

- The organic trace nutrients may be amino acids, vitamins, fatty acids and nucleic acids as they are or as contained in peptone, casamino acid, yeast extract, soy protein hydrolysate and the like. In cases of using an auxotroph the nutrient required for its growth should be supplemented.

The inorganic salts may be phosphate, magnesium salts, calcium salts, iron salts, manganese salts and the like.

Cultivation is conducted at a fermentation temperature from 20 to 45°C at a pH controlled to be in a range of from 5 to 9 with aeration. When the pH is controlled during the cultivation, calcium carbonate or alkali such as ammonia gas may be added for neutralization. After culturing for from 10 hours to 4 days, a significant amount of L-glutamic acid is accumulated in the culture medium.

L-glutamic acid in the culture medium after cultivation may be recovered by any of the known methods. For example, the cells are removed from the culture medium, which is then concentrated and precipitated or subjected to ion exchange chromatography to obtain L-glutamic acid.

#### Brief Description of the Drawings

Fig. 1 shows the construction procedure of pBR-sucAB.

Fig. 2 shows a procedure for disrupting the *sucA* gene on the chromosomal DNA of *Escherichia coli* W3110, and

Fig. 3 shows the construction procedure of pGK.

#### Examples

The present invention is further described by the following examples.

#### Example 1

##### (1) Cloning of *sucA* gene and dihydrolipoamide succinyl transferase gene of *Escherichia coli*

The nucleotide sequences of *sucA* gene and dihydrolipoamide succinyl transferase gene (hereinafter referred to as *sucB* gene) of *Escherichia coli* K12 are known. The known nucleotide sequences of *sucA* gene and *sucB* gene are disclosed in Euro. J. Biochem., Vol. 141, pp. 351 to 374 (1984), and also shown here as Sequ ID No. 7 in the sequence listing. The nucleotide sequence from the 327th through the 3128th base residues corresponds to ORF (open reading frame) of the *sucA* gene, while that from the 3143rd through the 4357th base residues corresponds to ORF of the *sucB* gene. According to the nucleotide sequences reported, primers shown in Sequ ID No. 1 to 4 were synthesized and *sucA* and *sucB* genes were amplified by PCR method employing the chromosomal DNA of *Escherichia coli* W3110 as a template.

The synthetic primers used to amplify the *sucA* gene had the nucleotide sequences shown in Sequ ID No. 1 and 2, and Sequ ID No. 1 corresponds to the sequence consisting of the 45th through the 65th base residues in the nucleotide sequence figure of the *sucA* gene described in Euro. J. Biochem., Vol. 141, p. 354 (1984). It also corresponds to the sequence consisting of the 45th through the 65th base residues of the nucleotide sequence shown as Sequ ID No. 7.

Sequ ID No. 2 corresponds to the sequence consisting of the 3173rd through the 3193rd base residues in the nucleotide sequence figure of the *sucB* gene shown in Euro. J. Biochem., Vol. 141, p. 364 (1984). It also corresponds to the sequence consisting of the 3173rd through the 3193rd base residues of the nucleotide sequence shown as Sequ ID No. 7.

The synthetic primers used to amplify the *sucB* gene had the nucleotide sequences shown in Sequ ID No. 3 and 4, and Sequ ID No. 3 corresponds to the sequence consisting of the 2179th through 2198th base residues in the nucleotide sequence figure of the *sucA* gene shown in Euro. J. Biochem., Vol. 141, p. 354 (1984). It also corresponds to the sequence consisting of the 2179th through the 2198th base residues of the nucleotide sequence shown as Sequ No. 7.

Sequ ID No. 4 corresponds to the sequence consisting of the 4566th through the 4591st base residues in the nucleotide sequence figure of the *sucB* gene shown in Euro. J. Biochem., Vol. 141, p. 364 (1984). It also corresponds to the sequence consisting of the 4566th through the 4591st base residues of the nucleotide sequence shown as Sequ ID No. 7. The *sucA* gene and the *sucB* gene form an operon.

The chromosomal DNA of *Escherichia coli* W3110 was recovered by a standard method (Seibutsukogaku Jikkensho, Ed. by Nippon Seibutsu Kogaku Kai, pp. 97 to 98, Baifukan (1992)).

The PCR reaction was carried out under the standard conditions described on page 8 of PCR Technology (Ed. by Henry Erlich, Stockton Press (1989)).

Both ends of PCR products thus produced were converted into blunt ends using T4 DNA polymerase and cloned into a vector pBR322 at the *EcoRV* site. The plasmid obtained by cloning the *sucA* gene into pBR322 was designated as pBR-sucA, and that constructed with *sucB* was designated as pBR-sucB. The plasmids

thus obtained were introduced into *Escherichia coli* JM109 and the plasmids were prepared. Then the restriction maps were constructed and compared with the restriction maps of the *sucA* and *sucB* genes reported, thereby confirming that the genes which had been cloned were the *sucA* and *sucB* genes.

As shown in Fig. 1, pBR-sucB was digested with *Kpn*I and *Eco*RI to prepare a DNA fragment containing the *sucB* gene. pBR-sucA was digested with *Kpn*I and *Eco*RI to prepare a large fragment. Both fragments were ligated using T4 DNA ligase to produce pBR-sucAB.

## (2) Disruption of the *sucA* gene on chromosomal DNA of *Escherichia coli* W3110

Fig. 2 outlines the disruption of the *sucA* gene on the chromosomal DNA of *Escherichia coli* W3110.

pBR-sucAB was digested with *Kpn*I and T4 DNA polymerase was used to obtain blunt ends. On the other hand, pUC4K (purchased from Pharmacia) was digested with *Pst*I to prepare a DNA fragment containing a kanamycin-resistance gene, which was converted to have blunt ends using T4 DNA polymerase. Both fragments were ligated using T4 DNA ligase to obtain pBR-sucA::Km<sup>r</sup>. From this plasmid, a *Hind*III-*Eco*RI fragment containing the kanamycin-resistance gene was cut out as a linear DNA, which was used to transform *Escherichia coli* JC7623 (*thr*-1, *ara*-14, *leu*B6,  $\Delta$ (*gpt*-*proA*)62, *lac*Y1, *tsx*-23, *sup*E44, *gal*1K2,  $\lambda^-$ , *rac*-, *sbc*B15, *his*G4, *rfb*D1, *rec*B21, *rec*C22, *rps*L31, *kdg*K51, *xyl*-5, *mtl*-1, *arg*E3, *thi*-1) obtained from the *Escherichia coli* Genetic Stock Center (at Yale University, USA), and strains in which the *sucA* gene on the chromosomal DNA was replaced with the *sucA* gene into which the kanamycin-resistance gene had been inserted (*sucA*::Km<sup>r</sup>) were screened on L medium (bactotrypton 10 g/l, yeast extract 5 g/l, NaCl 5 g/l, agar 15 g/l, pH 7.2) supplemented with 25  $\mu$ g/ml of kanamycin. Since *Escherichia coli* JC7623 possessed *recB*, *recC* and *sbcB*, recombination can be achieved at a high frequency even if the transformation is conducted using a linear DNA.

From each of twelve (12) kanamycin-resistant strains thus obtained, the chromosomal DNA was prepared and digested with *Kpn*I. By southern hybridization using a DNA fragment containing the *sucA* gene as a probe, all of 12 strains were confirmed to be strains in which the *sucA* gene on the chromosomal DNA was replaced with the *sucA* gene into which kanamycin-resistance gene had been inserted. While a wild strain exhibits two bands at 5.2 Kb and 6.2 Kb due to the presence of *Kpn*I site in the DNA fragment containing the *sucA* gene when a 2.6 Kb *Eco*RI-*Hind*III fragment containing the *sucA* gene of pBR-sucA was used as the probe in the southern hybridization, strains having the replacement with *sucA* gene into which kanamycin-resistance gene has been inserted exhibits only one band at 11.4 Kb due to the disruption of the *Kpn*I site upon introduction of the kanamycin-resistance gene. The kanamycin-resistance *Escherichia coli* JC7623 (*sucA*::Km<sup>r</sup>) thus obtained was then infected with P1 phage and the phage lysate was prepared. Then *Escherichia coli* W3100 strain was transduced with the *sucA*::Km<sup>r</sup>. Transduction with P1 phage was conducted by a standard method (Seibutsu-kogaku Jikkensho, Ed. by Nippon Seibutsu Kogaku Kai, pp. 75 to 76, Baifukan (1992)). The representative of the kanamycin-resistance strains isolated was designated as W3110 *sucA*::Km<sup>r</sup>.

The  $\alpha$ -KGDH activities of the strain W3110 *sucA*::Km<sup>r</sup> and *Escherichia coli* W3110 were determined according to the method by Reed et al (Methods in Enzymology, Vol. 13, pp. 55 (1969)). The results are shown in Table 1.  $\alpha$ -KGDH activity of *Escherichia coli* W3110 *sucA*::Km<sup>r</sup> was not detected. Thus, *Escherichia coli* W3110 *sucA*::Km<sup>r</sup> is a strain deficient in  $\alpha$ -KGDH activity.

Table 1

	W3110	W3110 <i>sucA</i> ::Km <sup>r</sup>
$\alpha$ -KGDH activity	3.70	Not detected
(Unit : micromoles/mg protein/min)		

## (3) Cloning of *gdhA* gene of *Escherichia coli* W3110

Similarly as in the cloning of the *sucA* and *sucB* genes, the PCR method was used to clone the *gdhA* gene. According to the nucleotide sequence of *gdhA* gene reported by Fernando et al, primers for PCR were synthesized. The nucleotide sequence of the *gdhA* gene is disclosed in Gene, Vol. 27, pp.193 to 199 (1984), and is also shown here as Sequ ID No. 8 in the sequence listings. The nucleotide sequences of the primers are shown in Sequ ID Nos. 5 and 6.

Sequ ID No. 5 corresponds to the sequence from the 191st through the 171st base residues in the nucleotide sequence figure of *gdhA* gene shown in Gene, Vol. 27, p.195 (1984), and it also corresponds to the

sequence from the 3rd through the 23rd base residues in Sequ ID No. 8.

Sequ ID No. 6 corresponds to the sequence consisting of the 1687th through the 1707th base residues in the nucleotide sequence figure of the *gdhA* gene shown in Gene, Vol. 27, p.195, (1984), and it also corresponds to the sequence consisting of 1880th through the 1900th base residues in Sequ ID No. 8.

Using the synthetic primers the *gdhA* gene was amplified by the PCR method employing the chromosomal DNA of *Escherichia coli* W3110 as a template. PCR products thus obtained were purified and converted to have blunt ends using T4 DNA polymerase, and then ligated to pBR 322 digested with *EcoRV* to obtain a plasmid pBRGDH.

#### (4) Construction of a plasmid having the *ppc* and *gdhA* genes

Fig. 3 shows the procedure for the construction of a plasmid having the *ppc* and *gdhA* genes. The plasmid pS2 in which 4.4Kb *Sall* fragment containing the whole region of the *ppc* gene derived from *Escherichia coli* K-12 was inserted into the *Sall* site of pBR322 (J. Biochem, Vol. 9, pp.909 to 916 (1984)) was digested with *HindIII* and both ends were made blunt using T4 DNA polymerase. On the other hand, a DNA fragment containing the *gdhA* gene synthesized by the PCR method was converted to have blunt ends using T4 DNA polymerase. Subsequently, both fragments were ligated using T4 DNA ligase. The plasmid thus obtained was used to transform a GDH deficient strain, *Escherichia coli* PA 340 (*thr-1*, *fhuA2*, *leuB6*, *lacY1*, *supE44*, *gal-6*,  $\lambda$ -, *gdh-1*, *hisG1*, *rfaD1*, *galP63*,  $\Delta$ (*gltB-F*), *rpsL19*, *malT1*( $\lambda$ ), *xy1-7*, *mtl-2*, *argH1*, *thi-1*) obtained from the *Escherichia coli* Genetic Stock Center (at Yale University, USA) and an ampicillin-resistant strain which had lost its glutamic acid requirement for growth was isolated. From this strain, a plasmid was prepared and the restriction map was constructed, whereby it was confirmed that the *ppc* and *gdhA* genes were present on this plasmid. This plasmid was designated as pGK.

#### (5) Introduction of pS2, pBRGDH and pGK into $\alpha$ -KGDH deficient strain *Escherichia coli* W3100 *sucA::Km<sup>r</sup>* and evaluation of L-glutamic acid-production

The  $\alpha$ -KGDH-deficient strain, *Escherichia coli* W3100 *sucA::Km<sup>r</sup>* was transformed with each of pS2, pBRGDH and pGK, and each of the transformed strains was inoculated into a 500-ml shaker flask containing 20 ml of the culture medium having the composition shown in Table 2. Cultivation was then carried out at 37 °C until the glucose in the culture medium was consumed completely. The results are shown in Table 3.

Table 2

Component	Concentration (g/l)
Glucose	40
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	20
KH <sub>2</sub> PO <sub>4</sub>	1
MgSO <sub>4</sub> ·7H <sub>2</sub> O	1
FeSO <sub>4</sub> ·7H <sub>2</sub> O	0.01
MnSO <sub>4</sub> ·5H <sub>2</sub> O	0.01
Yeast extract	2
Thiamine hydrochloride	0.01
CaCO <sub>3</sub>	50

Table 3

Strain	Accumulated L-glutamic acid (g/l)
W3110 <i>sucA::Km<sup>r</sup></i>	19.2
W3110 <i>sucA::Km<sup>r</sup>/pS2</i>	19.9
W3110 <i>sucA::Km<sup>r</sup>/pBRGDH</i>	2.8
W3110 <i>sucA::Km<sup>r</sup>/pGK (AJ 12949)</i>	23.3

Although the transformed strain having the PPC activity amplified by the introduction of pS2 exhibited slightly reduced growth as compared with the host strain, W3110 *sucA::Km<sup>r</sup>*, it accumulated L-glutamic acid in an amount similar to that accumulated by the host strain. The strain having GDH activity amplified by the introduction of pBRGDH exhibited quite poor growth, and the amount of the accumulated L-glutamic acid was surprisingly smaller than that accumulated by the strain W3110 *sucA::Km<sup>r</sup>*.

On the contrary, the transformed strain in which both of PPC and GDH activities were amplified simultaneously by the introduction of pGK exhibited growth similar to that of the host strain while producing an increased amount of accumulated L-glutamic acid. *Escherichia coli* W3110 *sucA::Km<sup>r</sup>* into which pGK plasmid having the *ppc* and *gdhA* genes had been introduced was designated as AJ 12949. *Escherichia coli* AJ 12949 was originally deposited under the accession number FERM P-14039 on December 28, 1993, at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken 305, Japan, and the deposit was converted into a deposit under the Budapest Treaty under the accession number FERM BP-4881 on November 11, 1994.

The host strain, namely, W3110 *sucA::Km<sup>r</sup>* can be obtained by curing the plasmid from the deposited strain, AJ 12949 without damaging the cell. The plasmid may be lost from AJ 12949 spontaneously, or may be cured in a curing procedure (Bact. Rev., Vol. 36, p.361 to 405 (1972)). An example of the curing procedure is as follows. The strain AJ 12949 is inoculated to the L-broth medium (Bactotrypton 10 g/l, yeast extract 5 g/l, NaCl 5 g/l, pH 7.2), and cultivated at 40°C overnight. The culture broth is diluted appropriately, and spread onto the L-medium. After incubating it at 37°C overnight, the colonies formed are transferred to the L-medium containing 100 µg/ml of ampicillin and then ampicillin-sensitive colonies are isolated. The strain thus obtained is W3110 *sucA::Km<sup>r</sup>*.

#### Advantages of the Invention

The method according to the present invention provides a mutant of the genus *Escherichia* having a higher productivity of L-glutamic acid as well as the efficient and low-cost method for the production of L-glutamic acid.

SEQUENCE LISTING

5

GENERAL INFORMATION:

APPLICANT:

NAME: Ajinomoto Co., Inc.  
10 STREET: 15-1, Kyobashi 1-chome  
CITY: Chuo-ku, Tokyo  
COUNTRY: Japan  
POSTAL CODE: none

15 TITLE OF INVENTION: Method of producing L-glutamic acid by fermentation

NUMBER OF SEQUENCES: 8

COMPUTER READABLE FORM:

20 MEDIUM TYPE: Diskette  
COMPUTER: IBM PC compatible  
OPERATING SYSTEM: MS-DOS

25

SEQUENCE DESCRIPTION:

SEQ ID No.: 1  
Length : 21 base pairs  
30 Type : Nucleotide  
Strandedness : Single  
Topology : Linear  
Molecule type: Synthetic DNA  
Feature : Primer for amplification of sucA gene of Escherichia coli  
Sequence  
35 ACGCGCAAGC GTCGCATCAG G 21

SEQ ID No.: 2  
40 Length : 21 base pairs  
Type : Nucleotide  
Strandedness : Single  
Topology : Linear  
Molecule type: Synthetic DNA  
Feature : Primer for amplification of sucA gene of Escherichia coli  
45 Sequence  
ATCGGCTACG AATTCAGGCA G 21

50 SEQ ID No.: 3  
Length : 20 base pairs  
Type : Nucleotide  
Strandedness : Single  
Topology : Linear  
55 Molecule type: Synthetic DNA  
Feature : Primer for amplification of sucB gene of Escherichia coli

Sequence  
 5 CCGGTCGCGG TACCTTCTTC 20

SEQ ID No.: 4  
 Length : 26 base pairs  
 Type : Nucleotide  
 Strandedness : Single  
 Topology : Linear  
 Molecule type: Synthetic DNA  
 Feature : Primer for amplification of sucB gene of Escherichia coli  
 Sequence  
 15 CGTAGACCGA ATTCTTCGTA TCGCTT 26

SQ ID No.: 5  
 Length : 21 base pairs  
 Type : Nucleotide  
 Strandedness : Single  
 Topology : Linear  
 Molecule type: Synthetic DNA  
 25 Feature : Primer for amplification of gdhA gene of Escherichia coli  
 Sequence  
 GGGTGGCAAA GCTTTAGCGT C 21

SEQ ID No.: 6  
 Length : 21 base pairs  
 Type : Nucleotide  
 Strandedness : Single  
 Topology : Linear  
 35 Molecule type: Synthetic DNA  
 Feature : Primer for amplification of gdhA gene of Escherichia coli  
 Sequence  
 TCGAGAAGCA TGCATTATAT A 21

SEQ ID No.: 7  
 Length : 4623 base pairs  
 Type : Nucleotide  
 45 Strandedness : Single  
 Topology : Linear  
 Molecule type: Genomic DNA  
 Original source  
 Organism : Escherichia coli  
 Features  
 50 Feature key : CDS                   ⇒from 327 to 318 bp coding sequence  
 Location : 327..3128  
 Method of feature determination : E  
 Feature key : CDS                   ⇒from 3143 to 4357 bp coding sequence  
 Location : 3143..4357  
 55 Method of feature determination : E

Sequence																
5	TCGATGTTGT	TGCAACGTAA	TGCGTAAACC	GTAGGCCTGA	TAAGACGCGC	AAGCGTCGCA										60
	TCAGGCAACC	AGTGCCGGAT	GCGCGTGAAC	GCCTTATCCG	GCCTACAAGT	CATTACCCGT										120
	AGGCCTGATA	AGCGCAGCGC	ATCAGGCGTA	ACAAAGAAAT	GCAGGAAATC	TTTAAAAACT										180
	GCCCCTGACA	CTAAGACAGT	TTTTAAAGGT	TCCTTCGCGA	GCCACTACGT	AGACAAGAGC										240
	TCGCAAGTGA	ACCCCGGCAC	GCACATCACT	GTGCGTGGTA	GTATCCACGG	CGAAGTAAGC										300
	ATAAAAAAGA	TGCTTAAGGG	ATCACG	ATG	CAG	AAC	AGC	GCT	TTG	AAA	GCC	TGG				353
				Met	Gln	Asn	Ser	Ala	Leu	Lys	Ala	Trp				
10				1												
	TTG	GAC	TCT	TCT	TAC	CTC	TCT	GGC	GCA	AAC	CAG	AGC	TGG	ATA	GAA	CAG
	Leu	Asp	Ser	Ser	Tyr	Leu	Ser	Gly	Ala	Asn	Gln	Ser	Trp	Ile	Glu	Gln
	10					15					20				25	
	CTC	TAT	GAA	GAC	TTC	TTA	ACC	GAT	CCT	GAC	TCG	GTT	GAC	GCT	AAC	TGG
	Leu	Tyr	Glu	Asp	Phe	Leu	Thr	Asp	Pro	Asp	Ser	Val	Asp	Ala	Asn	Trp
					30						35				40	
15	CGT	TCG	ACG	TTC	CAG	TTA	CCT	GGT	ACG	GGA	GTC	AAA	CCG	GAT	CAA	
	Arg	Ser	Thr	Phe	Gln	Gln	Leu	Pro	Gly	Thr	Gly	Val	Lys	Pro	Asp	Gln
				45				50					55			
	TTC	CAC	TCT	CAA	ACG	CGT	GAA	TAT	TTC	CGC	CGC	CTG	GCG	AAA	GAC	GCT
	Phe	His	Ser	Gln	Thr	Arg	Glu	Tyr	Phe	Arg	Arg	Leu	Ala	Lys	Asp	Ala
			60					65				70				
20	TCA	CGT	TAC	TCT	TCA	ACG	ATC	TCC	GAC	CCT	GAC	ACC	AAT	GTG	AAG	CAG
	Ser	Arg	Tyr	Ser	Ser	Thr	Ile	Ser	Asp	Pro	Asp	Thr	Asn	Val	Lys	Gln
		75					80				85					
	GTT	AAA	GTC	CTG	CAG	CTC	ATT	AAC	GCA	TAC	CGC	TTC	CGT	GGT	CAC	CAG
	Val	Lys	Val	Leu	Gln	Leu	Ile	Asn	Ala	Tyr	Arg	Phe	Arg	Gly	His	Gln
		90				95					100				105	
25	CAT	GCG	AAT	CTC	GAT	CCG	CTG	GGA	CTG	TGG	CAG	CAA	GAT	AAA	GTG	GCC
	His	Ala	Asn	Leu	Asp	Pro	Leu	Gly	Leu	Trp	Gln	Gln	Asp	Lys	Val	Ala
				110							115				120	
	GAT	CTG	GAT	CCG	TCT	TTC	CAC	GAT	CTG	ACC	GAA	GCA	GAC	TTC	CAG	GAG
	Asp	Leu	Asp	Pro	Ser	Phe	His	Asp	Leu	Thr	Glu	Ala	Asp	Phe	Gln	Glu
				125							130				135	
30	ACC	TTC	AAC	GTC	GGT	TCA	TTT	GCC	AGC	GGC	AAA	GAA	ACC	ATG	AAA	CTC
	Thr	Phe	Asn	Val	Gly	Ser	Phe	Ala	Ser	Gly	Lys	Glu	Thr	Met	Lys	Leu
			140					145					150			
	GGC	GAG	CTG	CTG	GAA	GCC	CTC	AAG	CAA	ACC	TAC	TGC	GGC	CCG	ATT	GGT
	Gly	Glu	Leu	Leu	Glu	Ala	Leu	Lys	Gln	Thr	Tyr	Cys	Gly	Pro	Ile	Gly
		155					160					165				
35	GCC	GAG	TAT	ATG	CAC	ATT	ACC	AGC	ACC	GAA	GAA	AAA	CGC	TGG	ATC	CAA
	Ala	Glu	Tyr	Met	His	Ile	Thr	Ser	Thr	Glu	Glu	Lys	Arg	Trp	Ile	Gln
		170				175					180				185	
	CAG	CGT	ATC	GAG	TCT	GGT	CGC	GCG	ACT	TTC	AAT	AGC	GAA	GAG	AAA	AAA
	Gln	Arg	Ile	Glu	Ser	Gly	Arg	Ala	Thr	Phe	Asn	Ser	Glu	Glu	Lys	Lys
				190							195				200	
40	CGC	TTC	TTA	AGC	GAA	CTG	ACC	GCC	GCT	GAA	GGT	CTT	GAA	CGT	TAC	CTC
	Arg	Phe	Leu	Ser	Glu	Leu	Thr	Ala	Ala	Glu	Gly	Leu	Glu	Arg	Tyr	Leu
				205						210				215		
	GGC	GCA	AAA	TTC	CCT	GGC	GCA	AAA	CGC	TTC	TCG	CTG	GAA	GGC	GGT	GAC
	Gly	Ala	Lys	Phe	Pro	Gly	Ala	Lys	Arg	Phe	Ser	Leu	Glu	Gly	Gly	Asp
			220					225					230			
45	GCG	TTA	ATC	CCG	ATG	CTT	AAA	GAG	ATG	ATC	CGC	CAC	GCT	GGC	AAC	AGC
	Ala	Leu	Ile	Pro	Met	Leu	Lys	Glu	Met	Ile	Arg	His	Ala	Gly	Asn	Ser
		235					240					245				
	GGC	ACC	CGC	GAA	GTG	GTT	CTC	GGG	ATG	GCG	CAC	CGT	GGT	CGT	CTG	AAC
	Gly	Thr	Arg	Glu	Val	Val	Leu	Gly	Met	Ala	His	Arg	Gly	Arg	Leu	Asn
		250				255					260				265	
50	GTG	CTG	GTG	AAC	GTG	GGT	AAA	AAA	CCG	CAA	GAC	TTG	TTC	GAC	GAG	
	Val	Leu	Val	Asn	Val	Leu	Gly	Lys	Lys	Pro	Gln	Asp	Leu	Phe	Asp	Glu
				270						275				280		
	TTC	GCC	GGT	AAA	CAT	AAA	GAA	CAC	CTC	GGC	ACG	GGT	GAC	GTG	AAA	TAC
	Phe	Ala	Gly	Lys	His	Lys	Glu	His	Leu	Gly	Thr	Gly	Asp	Val	Lys	Tyr
55																

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			285				290				295						
5	CAC	ATG	GGC	TTC	TCG	TCT	GAC	TTC	CAG	ACC	GAT	GGC	GGC	CTG	GTG	CAC	1265
	His	Met	Gly	Phe	Ser	Ser	Asp	Phe	Gln	Thr	Asp	Gly	Gly	Leu	Val	His	
			300					305				310					
	CTG	GCG	CTG	GCG	TTT	AAC	CCG	TCT	CAC	CTT	GAG	ATT	GTA	AGC	CCG	GTA	1313
	Leu	Ala	Leu	Ala	Phe	Asn	Pro	Ser	His	Leu	Glu	Ile	Val	Ser	Pro	Val	
			315				320					325					
10	GTT	ATC	GGT	TCT	GTT	CGT	GCC	CGT	CTG	GAC	AGA	CTT	GAT	GAG	CCG	AGC	1361
	Val	Ile	Gly	Ser	Val	Arg	Ala	Arg	Leu	Asp	Arg	Leu	Asp	Glu	Pro	Ser	
			330			335					340					345	
	AGC	AAC	AAA	GTG	CTG	CCA	ATC	ACC	ATC	CAC	GGT	GAC	GCC	GCA	GTG	ACC	1409
	Ser	Asn	Lys	Val	Leu	Pro	Ile	Thr	Ile	His	Gly	Asp	Ala	Ala	Val	Thr	
				350						355					360		
15	GGG	CAG	GGC	GTG	GTT	CAG	GAA	ACC	CTG	AAC	ATG	TCG	AAA	GCG	CGT	GGT	1457
	Gly	Gln	Gly	Val	Val	Gln	Glu	Thr	Leu	Asn	Met	Ser	Lys	Ala	Arg	Gly	
			365						370					375			
	TAT	GAA	GTT	GGC	GGT	ACG	GTA	CGT	ATC	GTT	ATC	AAC	AAC	CAG	GTT	GGT	1505
	Tyr	Glu	Val	Gly	Gly	Thr	Val	Arg	Ile	Val	Ile	Asn	Asn	Gln	Val	Gly	
			380					385					390				
20	TTC	ACC	ACC	TCT	AAT	CCG	CTG	GAT	GCC	CGT	TCT	ACG	CCG	TAC	TGT	ACT	1553
	Phe	Thr	Thr	Ser	Asn	Pro	Leu	Asp	Ala	Arg	Ser	Thr	Pro	Tyr	Cys	Thr	
			395				400					405					
	GAT	ATC	GGT	AAG	ATG	GTT	CAG	GCC	CCG	ATT	TTC	CAC	GTT	AAC	GCG	GAC	1601
	Asp	Ile	Gly	Lys	Met	Val	Gln	Ala	Pro	Ile	Phe	His	Val	Asn	Ala	Asp	
			410			415					420				425		
25	GAT	CCG	GAA	GCC	GTT	GCC	TTT	GTG	ACC	CGT	CTG	GCG	CTC	GAT	TTC	CGT	1649
	Asp	Pro	Glu	Ala	Val	Ala	Phe	Val	Thr	Arg	Leu	Ala	Leu	Asp	Phe	Arg	
				430						435					440		
	AAC	ACC	TTT	AAA	CGT	GAT	GTC	TTC	ATC	GAC	CTG	GTG	TCG	TAC	CGC	CGT	1697
	Asn	Thr	Phe	Lys	Arg	Asp	Val	Phe	Ile	Asp	Leu	Val	Ser	Tyr	Arg	Arg	
			445					450						455			
30	CAC	GGC	CAC	AAC	GAA	GCC	GAC	GAG	CCG	AGC	GCA	ACC	CAG	CCG	CTG	ATG	1745
	His	Gly	His	Asn	Glu	Ala	Asp	Glu	Pro	Ser	Ala	Thr	Gln	Pro	Leu	Met	
			460					465					470				
	TAT	CAG	AAA	ATC	AAA	AAA	CAT	CCG	ACA	CCG	CGC	AAA	ATC	TAC	GCT	GAC	1793
	Tyr	Gln	Lys	Ile	Lys	Lys	His	Pro	Thr	Pro	Arg	Lys	Ile	Tyr	Ala	Asp	
			475				480					485					
35	AAG	CTG	GAG	CAG	GAA	AAA	GTG	GCG	ACG	CTG	GAA	GAT	GCC	ACC	GAG	ATG	1841
	Lys	Leu	Glu	Gln	Glu	Lys	Val	Ala	Thr	Leu	Glu	Asp	Ala	Thr	Glu	Met	
			490			495					500				505		
	GTT	AAC	CTG	TAC	CGC	GAT	GCG	CTG	GAT	GCT	GGC	GAT	TGC	GTA	GTG	GCA	1889
	Val	Asn	Leu	Tyr	Arg	Asp	Ala	Leu	Asp	Ala	Gly	Asp	Cys	Val	Val	Ala	
				510						515					520		
40	GAG	TGG	CGT	CCG	ATG	AAC	ATG	CAC	TCT	TTC	ACC	TGG	TCG	CCG	TAC	CTC	1937
	Glu	Trp	Arg	Pro	Met	Asn	Met	His	Ser	Phe	Thr	Trp	Ser	Pro	Tyr	Leu	
				525					530					535			
	AAC	CAC	GAA	TGG	GAC	GAA	GAG	TAC	CCG	AAC	AAA	GTT	GAG	ATG	AAG	CGC	1985
	Asn	His	Glu	Trp	Asp	Glu	Glu	Tyr	Pro	Asn	Lys	Val	Glu	Met	Lys	Arg	
			540					545					550				
45	CTG	CAG	GAG	CTG	GCG	AAA	CGC	ATC	AGC	ACG	GTG	CCG	GAA	GCA	GTT	GAA	2033
	Leu	Gln	Glu	Leu	Ala	Lys	Arg	Ile	Ser	Thr	Val	Pro	Glu	Ala	Val	Glu	
			555				560					565					
	ATG	CAG	TCT	CGC	GTT	GCC	AAG	ATT	TAT	GGC	GAT	CGC	CAG	GCG	ATG	GCT	2081
	Met	Gln	Ser	Arg	Val	Ala	Lys	Ile	Tyr	Gly	Asp	Arg	Gln	Ala	Met	Ala	
			570			575					580				585		
50	GCC	GGT	GAG	AAA	CTG	TTC	GAC	TGG	GCG	GGT	GCG	GAA	AAC	CTC	GCT	TAC	2129
	Ala	Gly	Glu	Lys	Leu	Phe	Asp	Trp	Gly	Gly	Ala	Glu	Asn	Leu	Ala	Tyr	
				590						595					600		
	GCC	ACG	CTG	GTT	GAT	GAA	GCG	ATT	CCG	GTT	CGC	CTG	TCG	GGT	GAA	GAC	2177
	Ala	Thr	Leu	Val	Asp	Glu	Gly	Ile	Pro	Val	Arg	Leu	Ser	Gly	Glu	Asp	
				605					610					615			
55	TCC	GGT	CGC	GGT	ACC	TTC	TTC	CAC	CGC	CAC	GCG	GTG	ATC	CAC	AAC	CAG	2225

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5	Ser	Gly	Arg	Gly	Thr	Phe	Phe	His	Arg	His	Ala	Val	Ile	His	Asn	Gln	
			620					625					630				
	TCT	AAC	GGT	TCC	ACT	TAC	ACG	CCG	CTG	CAA	CAT	ATC	CAT	AAC	GGG	CAG	2273
	Ser	Asn	Gly	Ser	Thr	Tyr	Thr	Pro	Leu	Gln	His	Ile	His	Asn	Gly	Gln	
			635					640					645				
	GGC	CCG	TTC	CGT	GTC	TGG	GAC	TCC	GTA	CTG	TCT	GAA	GAA	GCA	GTG	CTG	2321
	Gly	Ala	Phe	Arg	Val	Trp	Asp	Ser	Val	Leu	Ser	Glu	Glu	Ala	Val	Leu	
10			650				655				660					665	
	GCG	TTT	GAA	TAT	GGT	TAT	GCC	ACC	GCA	GAA	CCA	CGC	ACT	CTG	ACC	ATC	2369
	Ala	Phe	Glu	Tyr	Gly	Tyr	Ala	Thr	Ala	Glu	Pro	Arg	Thr	Leu	Thr	Ile	
					670					675					680		
	TGG	GAA	GCG	CAG	TTC	GGT	GAC	TTC	GCC	AAC	GGT	GCG	CAG	GTG	GTT	ATC	2417
	Trp	Glu	Ala	Gln	Phe	Gly	Asp	Phe	Ala	Asn	Gly	Ala	Gln	Val	Val	Ile	
15					685					690					695		
	GAC	CAG	TTC	ATC	TCC	TCT	GGC	GAA	CAG	AAA	TGG	GCG	CGG	ATG	TGT	GGT	2465
	Asp	Gln	Phe	Ile	Ser	Ser	Gly	Glu	Gln	Lys	Trp	Gly	Arg	Met	Cys	Gly	
			700					705						710			
	CTG	GTG	ATG	TTG	CTG	CCG	CAC	GGT	TAC	GAA	GGG	CAG	GGG	CCG	GAG	CAC	2513
	Leu	Val	Met	Leu	Leu	Pro	His	Gly	Tyr	Glu	Gly	Gln	Gly	Pro	Glu	His	
20							720						725				
	TCC	TCC	GCG	CGT	CTG	GAA	CGT	TAT	CTG	CAA	CTT	TGT	GCT	GAG	CAA	AAC	2561
	Ser	Ser	Ala	Arg	Leu	Glu	Arg	Tyr	Leu	Gln	Leu	Cys	Ala	Glu	Gln	Asn	
							735					740				745	
	ATG	CAG	GTT	TGC	GTA	CCG	TCT	ACC	CCG	GCA	CAG	GTT	TAC	CAC	ATG	CTG	2609
	Met	Gln	Val	Cys	Val	Pro	Ser	Thr	Pro	Ala	Gln	Val	Tyr	His	Met	Leu	
25							750				755					760	
	CGT	CGT	CAG	GCG	CTG	CGC	GGG	ATG	CGT	CGT	CCG	CTG	GTC	GTG	ATG	TCG	2657
	Arg	Arg	Gln	Ala	Leu	Arg	Gly	Met	Arg	Arg	Pro	Leu	Val	Val	Met	Ser	
							765			770					775		
	CCG	AAA	TCC	CTG	CTG	CGT	CAT	CCG	CTG	GCG	GTT	TCC	AGC	CTC	GAA	GAA	2705
	Pro	Lys	Ser	Leu	Leu	Arg	His	Pro	Leu	Ala	Val	Ser	Ser	Leu	Glu	Glu	
30								785					790				
	CTG	GCG	AAC	GGC	ACC	TTC	CTG	CCA	GCC	ATC	GGT	GAA	ATC	GAC	GAG	CTT	2753
	Leu	Ala	Asn	Gly	Thr	Phe	Leu	Pro	Ala	Ile	Gly	Glu	Ile	Asp	Glu	Leu	
							800						805				
	GAT	CCG	AAG	GGC	GTG	AAG	CGC	GTA	GTG	ATG	TGT	TCT	GGT	AAG	GTT	TAT	2801
	Asp	Pro	Lys	Gly	Val	Lys	Arg	Val	Val	Met	Cys	Ser	Gly	Lys	Val	Tyr	
35							815					820				825	
	TAC	GAC	CTG	CTG	GAA	CAG	CGT	CGT	AAG	AAC	AAT	CAA	CAC	GAT	GTC	GCC	2849
	Tyr	Asp	Leu	Leu	Glu	Gln	Arg	Arg	Lys	Asn	Asn	Gln	His	Asp	Val	Ala	
						830				835					840		
	ATT	GTG	CGT	ATC	GAG	CAA	CTC	TAC	CCG	TTC	CCG	CAT	AAA	GCG	ATG	CAG	2897
	Ile	Val	Arg	Ile	Glu	Gln	Leu	Tyr	Pro	Phe	Pro	His	Lys	Ala	Met	Gln	
40							845			850					855		
	GAA	GTG	TTG	CAG	CAG	TTT	GCT	CAC	GTC	AAG	GAT	TTT	GTC	TGG	TGC	CAG	2945
	Glu	Val	Leu	Gln	Gln	Phe	Ala	His	Val	Lys	Asp	Phe	Val	Trp	Cys	Gln	
							860			865				870			
	GAA	GAG	CCG	CTC	AAC	CAG	GGC	GCA	TGG	TAC	TGC	AGC	CAG	CAT	CAT	TTC	2993
	Glu	Glu	Pro	Leu	Asn	Gln	Gly	Ala	Trp	Tyr	Cys	Ser	Gln	His	His	Phe	
45							875					885					
	CGT	GAA	GTG	ATT	CCG	TTT	GGG	GCT	TCT	CTG	CGT	TAT	GCA	GGC	CGC	CCG	3041
	Arg	Glu	Val	Ile	Pro	Phe	Gly	Ala	Ser	Leu	Arg	Tyr	Ala	Gly	Arg	Pro	
							895				900				905		
	GCC	TCC	GCC	TCT	CCG	GCG	GTA	GGG	TAT	ATG	TCC	GTT	CAC	CAG	AAA	CAG	3089
	Ala	Ser	Ala	Ser	Pro	Ala	Val	Gly	Tyr	Met	Ser	Val	His	Gln	Lys	Gln	
50							910				915					920	
	CAA	CAA	GAT	CTG	GTT	AAT	GAC	GCG	CTG	AAC	GTC	GAA	TAAATAAAGG				3135
	Gln	Gln	Asp	Leu	Val	Asn	Asp	Ala	Leu	Asn	Val	Glu					
							925										
	ATACACA	ATG	AGT	AGC	GTA	GAT	ATT	CTG	GTC	CCT	GAC	CTG	CCT	GAA	TCC		3184
55			Met	Ser	Ser	Val	Asp	Ile	Leu	Val	Pro	Asp	Leu	Pro	Glu	Ser	
			1					5					10				

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5	GTA	GCC	GAT	GCC	ACC	GTC	GCA	ACC	TGG	CAT	AAA	AAA	CCC	GGC	GAC	GCA	3232
	Val	Ala	Asp	Ala	Thr	Val	Ala	Thr	Trp	His	Lys	Lys	Pro	Gly	Asp	Ala	
10	GTC	GTA	CGT	GAT	GAA	GTG	CTG	GTA	GAA	ATC	GAA	ACT	GAC	AAA	GTG	GTA	3280
	Val	Val	Arg	Asp	Glu	Val	Leu	Val	Glu	Ile	Glu	Thr	Asp	Lys	Val	Val	
15	CTG	GAA	GTA	CCG	GCA	TCA	GCA	GAC	GGC	ATT	CTG	GAT	GCG	GTT	CTG	GAA	3328
	Leu	Glu	Val	Pro	Ala	Ser	Ala	Asp	Gly	Ile	Leu	Asp	Ala	Val	Leu	Glu	
20	GAT	GAA	GGT	ACA	ACG	GTA	ACG	TCT	CGT	CAG	ATC	CTT	GGT	GCG	CTG	CGT	3376
	Asp	Glu	Gly	Thr	Thr	Val	Thr	Ser	Arg	Gln	Ile	Leu	Gly	Arg	Leu	Arg	
25	GAA	GGC	AAC	AGC	GCC	GGT	AAA	GAA	ACC	AGC	GCC	AAA	TCT	GAA	GAG	AAA	3424
	Glu	Gly	Asn	Ser	Ala	Gly	Lys	Glu	Thr	Ser	Ala	Lys	Ser	Glu	Glu	Lys	
30	GCG	TCC	ACT	CCG	GCG	CAA	CGC	CAG	CAG	GCG	TCT	CTG	GAA	GAG	CAA	AAC	3472
	Ala	Ser	Thr	Pro	Ala	Gln	Arg	Gln	Gln	Ala	Ser	Leu	Glu	Glu	Gln	Asn	
35	AAC	GAT	GCG	TTA	AGC	CCG	GCG	ATC	CGT	CGC	CTG	GCT	GAA	CAC	AAT		3520
	Asn	Asp	Ala	Leu	Ser	Pro	Ala	Ile	Arg	Arg	Leu	Leu	Ala	Glu	His	Asn	
40	CTC	GAC	GCC	AGC	GCC	ATT	AAA	GGC	ACC	GGT	GTG	GGT	GGT	CGT	CTG	ACT	3568
	Leu	Asp	Ala	Ser	Ala	Ile	Lys	Gly	Thr	Gly	Val	Gly	Gly	Arg	Leu	Thr	
45	CGT	GAA	GAT	GTG	GAA	AAA	CAT	CTG	GCG	AAA	GCC	CCG	GCG	AAA	GAG	TCT	3616
	Arg	Glu	Asp	Val	Glu	Lys	His	Leu	Ala	Lys	Ala	Pro	Ala	Lys	Glu	Ser	
50	GCT	CCG	GCA	GCG	GCT	GCT	CCG	GCG	GCG	CAA	CCG	GCT	CTG	GCT	GCA	CGT	3664
	Ala	Pro	Ala	Ala	Ala	Ala	Pro	Ala	Ala	Gln	Pro	Ala	Leu	Ala	Ala	Arg	
55	AGT	GAA	AAA	CGT	GTC	CCG	ATG	ACT	CGC	CTG	CGT	AAG	CGT	GTG	GCA	GAG	3712
	Ser	Glu	Lys	Arg	Val	Pro	Met	Thr	Arg	Leu	Arg	Lys	Arg	Val	Ala	Glu	
60	CGT	CTG	CTG	GAA	GCG	AAA	AAC	TCC	ACC	GCC	ATG	CTG	ACC	ACG	TTC	AAC	3760
	Arg	Leu	Leu	Glu	Ala	Lys	Asn	Ser	Thr	Ala	Met	Leu	Thr	Thr	Phe	Asn	
65	GAA	GTC	AAC	ATG	AAG	CCG	ATT	ATG	GAT	CTG	CGT	AAG	CAG	TAC	GGT	GAA	3808
	Glu	Val	Asn	Met	Lys	Pro	Ile	Met	Asp	Leu	Arg	Lys	Gln	Tyr	Gly	Glu	
70	GCG	TTT	GAA	AAA	CGC	CAC	GGC	ATC	CGT	CTG	GGC	TTT	ATG	TCC	TTC	TAC	3856
	Ala	Phe	Glu	Lys	Arg	His	Gly	Ile	Arg	Leu	Gly	Phe	Met	Ser	Phe	Tyr	
75	GTG	AAA	GCG	GTG	GTT	GAA	GCC	CTG	AAA	CGT	TAC	CCG	GAA	STG	AAC	GCT	3904
	Val	Lys	Ala	Val	Val	Glu	Ala	Leu	Lys	Arg	Tyr	Pro	Glu	Val	Asn	Ala	
80	TCT	ATC	GAC	GGC	GAT	GAC	GTG	GTT	TAC	CAC	AAC	TAT	TTC	GAC	GTC	AGC	3952
	Ser	Ile	Asp	Gly	Asp	Asp	Val	Val	Tyr	His	Asn	Tyr	Phe	Asp	Val	Ser	
85	ATG	GCG	GTT	TCT	ACG	CCG	CGC	GGC	CTG	GTG	ACG	CCG	GTT	CTG	CGT	GAT	4000
	Met	Ala	Val	Ser	Thr	Pro	Arg	Gly	Leu	Val	Thr	Pro	Val	Leu	Arg	Asp	
90	GTC	GAT	ACC	CTC	GGC	ATG	GCA	GAC	ATC	GAG	AAG	AAA	ATC	AAA	GAG	CTG	4048
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95	GCA	GTC	AAA	GGC	CGT	GAC	GGC	AAG	CTG	ACC	GTT	GAA	GAT	CTG	ACC	GGT	4096
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100	GGT	AAC	TTC	ACC	ATC	ACC	AAC	GGT	GGT	GTG	TTC	GGT	TCC	CTG	ATG	TCT	4144
	Gly	Asn	Phe	Thr	Ile	Thr	Asn	Gly	Gly	Val	Phe	Gly	Ser	Leu	Met	Ser	
105	ACG	CCG	ATC	ATC	AAC	CCG	CCG	CAG	AGC	GCA	ATT	CTG	GGT	ATG	CAC	GCT	4192
	Thr	Pro	Ile	Ile	Asn	Pro	Pro	Gln	Ser	Ala	Ile	Leu	Gly	Met	His	Ala	

5 335 340 345 350  
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 370 375 380  
 10 GAA TCC GTG GGC TTC CTG GTA ACG ATC AAA GAG TTG CTG GAA GAT CCG 4336  
 Glu Ser Val Gly Phe Leu Val Thr Ile Lys Glu Leu Leu Glu Asp Pro  
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 Thr Arg Leu Leu Leu Asp Val  
 400 405  
 15 CCGGATAAGG CATTATCGCC TTCTCCGGCA ATTGAAGCCT GATGCGACGC TGACGCGTCT 4447  
 TATCAGGCCT ACGGGACCAC CAATGTAGGT CGGATAAGGC GCAACGCCGC ATCCGACAAG 4507  
 CGATGCCCTGA TGTGACGTTT AACGTGTCTT ATCAGGCCTA CGGGTGACCG ACAATGCCCG 4567  
 GAAGCGATAC GAAATATTTCG GTCTACGGTT TAAAGATAA CGATTACTGA AGGATG 4623

20 SEQ ID No.: 8  
 Length : 1937 base pairs  
 Type : Nucleotide  
 Strandedness : Single  
 Topology : Linear  
 25 Molecule type: Genomic DNA  
 Original source  
 Organism : Escherichia coli  
 Sequence feature  
 Feature key : CDS                   ⇒from 194 to 1537 bp coding sequence  
 Location : 194..1537  
 30 Method of feature determination : E  
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 GTCGAAACT GCAAAAGCAC ATGACATAAA CAACATAAGC ACAATCGTAT TAATATATAA 180  
 GGGTTTATA TCT ATG GAT CAG ACA TAT TCT CTG GAG TCA TTC CTC AAC 229  
 35 Met Asp Gln Thr Tyr Ser Leu Glu Ser Phe Leu Asn  
 1 5 10  
 CAT GTC CAA AAG CGC GAC CCG AAT CAA ACC GAG TTC GCG CAA GCC GTT 277  
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 15 20 25  
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 40 Arg Glu Val Met Thr Thr Leu Trp Pro Phe Leu Glu Gln Asn Pro Lys  
 30 35 40  
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 45 Ile Gln Phe Arg Val Val Trp Val Asp Asp Arg Asn Gln Ile Gln Val  
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 Leu Gly Phe Glu Gln Thr Phe Lys Asn Ala Leu Thr Thr Leu Pro Met  
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 55 Gly Gly Gly Lys Gly Gly Ser Asp Phe Asp Pro Lys Gly Lys Ser Glu

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5	Gly	Glu	Val	Met	Arg	Phe	Cys	Gln	Ala	Leu	Met	Thr	Glu	Leu	Tyr	Arg	
					145					150							
	CAC	CTG	GGC	GCG	GAT	ACC	GAC	GTT	CCG	GCA	GGT	GAT	ATC	GGG	GTT	GGT	709
	His	Leu	Gly	Ala	Asp	Thr	Asp	Val	Pro	Ala	Gly	Asp	Ile	Gly	Val	Gly	
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	GGT	CGT	GAA	GTC	GGC	TTT	ATG	GCG	GGG	ATG	ATG	AAA	AAG	CTC	TCC	AAC	757
10	Gly	Arg	Glu	Val	Gly	Phe	Met	Ala	Gly	Met	Met	Lys	Lys	Leu	Ser	Asn	
			175					180					185				
	AAT	ACC	GCC	TGC	GTC	TTC	ACC	GGT	AAG	GGC	CTT	TCA	TTT	GGC	GGC	AGT	805
	Asn	Thr	Ala	Cys	Val	Phe	Thr	Gly	Lys	Gly	Leu	Ser	Phe	Gly	Gly	Ser	
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15	Leu	Ile	Arg	Pro	Glu	Ala	Thr	Gly	Tyr	Gly	Leu	Val	Tyr	Phe	Thr	Glu	
		205				210					215					220	
	GCA	ATG	CTA	AAA	CGC	CAC	GGT	ATG	GGT	TTT	GAA	GGG	ATG	CGC	GTT	TCC	901
	Ala	Met	Leu	Lys	Arg	His	Gly	Met	Gly	Phe	Glu	Gly	Met	Arg	Val	Ser	
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20	Val	Ser	Gly	Ser	Gly	Asn	Val	Ala	Gln	Tyr	Ala	Ile	Glu	Lys	Ala	Met	
			240						245					250			
	GAA	TTT	GGT	GCT	CGT	GTG	ATC	ACT	GCG	TCA	GAC	TCC	AGC	GGC	ACT	GTA	997
	Glu	Phe	Gly	Ala	Arg	Val	Ile	Thr	Ala	Ser	Asp	Ser	Ser	Gly	Thr	Val	
		255					260					265					
	GTT	GAT	GAA	AGC	GGA	TTC	ACG	AAA	GAG	AAA	CTG	GCA	CGT	CTT	ATC	GAA	1045
25	Val	Asp	Glu	Ser	Gly	Phe	Thr	Lys	Glu	Lys	Leu	Ala	Arg	Leu	Ile	Glu	
		270				275					280						
	ATC	AAA	GCC	AGC	CGC	GAT	GGT	CGA	GTG	GCA	GAT	TAC	GCC	AAA	GAA	TTT	1093
	Ile	Lys	Ala	Ser	Arg	Asp	Gly	Arg	Val	Ala	Asp	Tyr	Ala	Lys	Glu	Phe	
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	GGT	CTG	GTC	TAT	CTC	GAA	GGC	CAA	CAG	CCG	TGG	TCT	CTA	CCG	GTT	GAT	1141
30	Gly	Leu	Val	Tyr	Leu	Glu	Gly	Gln	Gln	Pro	Trp	Ser	Leu	Pro	Val	Asp	
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	ATC	GCC	CTG	CCT	TGC	GCC	ACC	CAG	AAT	GAA	CTG	GAT	GTT	GAC	GCC	GCG	1189
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	CAT	CAG	CTT	ATC	GCT	AAT	GGC	GTT	AAA	GCC	GTC	GCC	GAA	GGG	GCA	AAT	1237
35	His	Gln	Leu	Ile	Ala	Asn	Gly	Val	Lys	Ala	Val	Ala	Glu	Gly	Ala	Asn	
			335					340					345				
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40	Leu	Phe	Ala	Pro	Gly	Lys	Ala	Ala	Asn	Ala	Gly	Gly	Val	Ala	Thr	Ser	
		365				370					375					380	
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45	Lys	Val	Asp	Ala	Arg	Leu	His	His	Ile	Met	Leu	Asp	Ile	His	His	Ala	
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	Cys	Val	Glu	His	Gly	Gly	Glu	Gly	Glu	Gln	Thr	Asn	Tyr	Val	Gln	Gly	
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	Gly	Val	Ile														
		445															
55	ACAAATGGGC	ACAATTCATT	GCAGTTACGC	TCTAATGTAG	GCCGGGCAAG	CGCAGCGCCC											1634

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5 CCGGCAAAAT TTCAGGCGTT TATGAGTATT TAACGGATGA TGCTCCCCAC GGAACATTTC 1694  
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 GCCTTTCGAG AAGCAAGCAT TATATAATGC CAGGCCAGTT CTTCTTCAAT TGTCCCGTTT 1934  
 TGA 1937

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# Claims

1. A mutant of the genus Escherichia having L-glutamic acid-productivity, said mutant having deficient or reduced  $\alpha$ -ketoglutarate dehydrogenase activity and enhanced phosphoenolpyruvate carboxylase and glutamate dehydrogenase activities.
2. A method of producing L-glutamic acid by fermentation comprising culturing in a liquid culture medium a mutant of the genus Escherichia having L-glutamic acid-productivity said mutant having deficient or reduced  $\alpha$ -ketoglutarate dehydrogenase activity and enhanced phosphoenolpyruvate carboxylase and glutamate dehydrogenase activities, accumulating L-glutamic acid in the culture, and recovering L-glutamic acid therefrom.

Fig.1

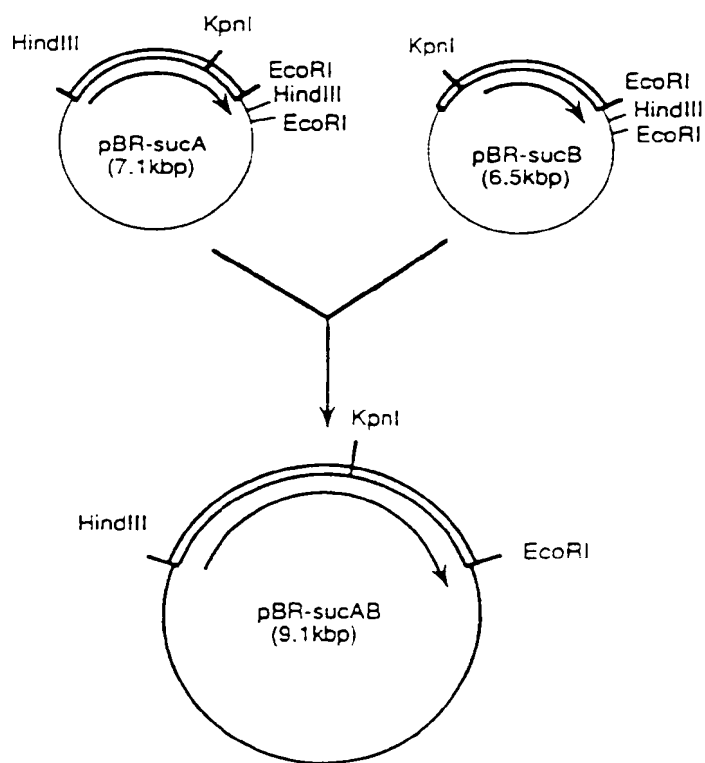


Fig. 2

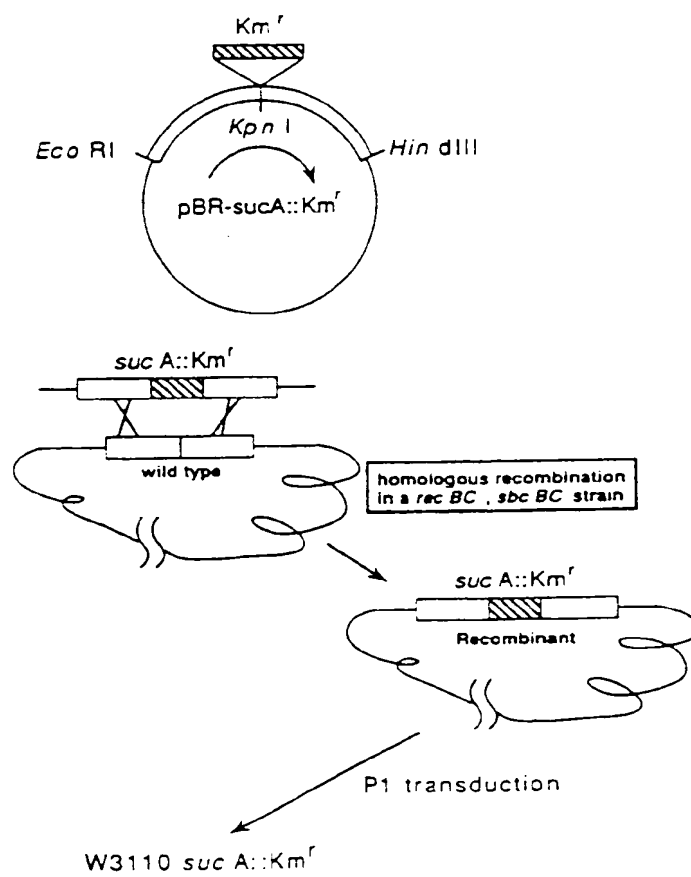
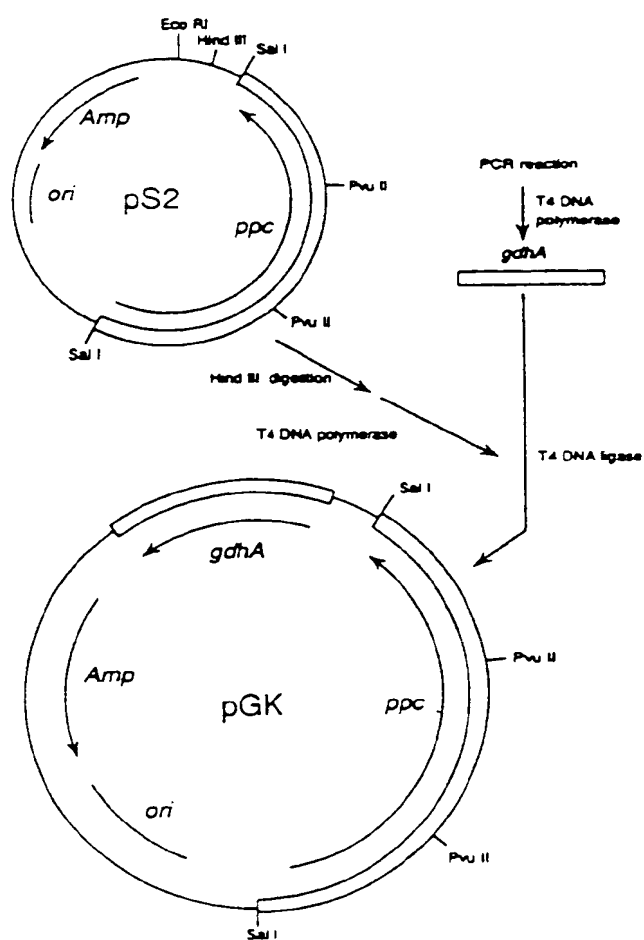


Fig.3



(19)



Europäisches Patentamt  
European Patent Office  
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(11)

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(12)

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**Kawasaki-ku, Kawasaki-shi, Kanagawa-ken (JP)**

(54) **Method of producing L-glutamic acid by fermentation**

(57) The present invention provides a low-cost and efficient production method of L-glutamic acid, by improving the productivity of L-glutamic acid in a microorganism of the genus Escherichia.

[Constitution]

A method of producing L-glutamic acid by fermentation

comprising culturing in a liquid culture medium a mutant of the genus Escherichia having L-glutamic acid-producing ability whose  $\alpha$ -ketoglutarate dehydrogenase activity is deficient or reduced and phosphoenolpyruvate carboxylase and glutamate dehydrogenase activities are amplified, accumulating L-glutamic acid in the culture, and recovering L-glutamic acid therefrom

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European Patent  
Office

# EUROPEAN SEARCH REPORT

Application Number  
EP 95 10 0259

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. CL.6)
D,A	FR 2 680 178 A (AJINOMOTO CO., INC.) 12 February 1993 * page 1, line 26 - page 3, line 8 *	1,2	C12N15/52 C12P13/14 C12N1/21 //(C12N1/21, C12R1:19)
A	EP 0 143 195 A (AJINOMOTO CO., INC.) 5 June 1985 * page 1, line 14 - page 4, line 14 *	1,2	
A	FR 2 575 492 A (ASAHI KASEI KOGYO KABUSHIKI KAISHA) 4 July 1986 * page 3, line 11 - line 33 * * page 7, line 9 - line 23 *	1,2	
			TECHNICAL FIELDS SEARCHED (Int. CL.6)
			C12P
The present search report has been drawn up for all claims			
Place of search		Date of completion of the search	Examiner
THE HAGUE		1 April 1997	Montero Lopez, B
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		I : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	

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